Abstract# 2917

MUTATION ARG2150 -> HIS IN THE FACTOR VIII C1 DOMAIN ALTERS THE BINDING OF FACTOR VIII TO VON WILLEBRAND FACTOR AND IS RESPONSIBLE FOR A MILD HEMOPHILIA A PHENOTYPE. M.G. Jacquemin[a], K. Peerlinck[b], J.G. Gilles, A. Benhida[a], B. Desqueper[a], R. Jacquemin[a], K. Peerlinck[b], J.G. Gilles, A. Benhida[a], B. Desqueper[a], R. Lavend'homme[a], J. Vermylen[a], J.M.R. Saint-Remy. *Center for Molecular and Vascular Biology, Katholieke Universiteit Leuven, Leuven, Belgium.*

Although mutations located in the factor VIII (FVII) C1 domain have been identified in patients with mild/moderate hemophilia A, the role of this domain in FVIII function or stability remains unknown. We have investigated the possible role of C1 in FVIII interaction with vWF by using a human monoclonal antibody recognising the C1 domain and plasma FVIII (~0.1 IU/ml) of mild hemophilia A patients' carrying the mutation Arg2150 -> His. An IgG4 human monoclonal antibody, LE2E9, was obtained by *in vitro* immortalisation of memory B lymphocytes of a mild hemophilia A patient (LE) who developed an immune response towards wild type FVIII but remained tolerant towards self FVIII carrying the mutation Arg2150 -> His. Epitope mapping with recombinant FVIII domains demonstrated that LE2E9 recognises only wild type FVIII C1 domain. LE2E9 and its Fab fragment completely inhibited the binding of wild type FVIII to vWF, suggesting that the C1 epitope recognised by this antibody may contribute to the binding of FVIII to vWF. Direct evidence that residue Arg2150 is important for the interaction of FVIII with vWF was obtained by demonstrating an alteration of the binding of patient LE's FVIII to vWF. When plasma vWF/FVIII complexes were captured on a Sepharose column coated with an anti-vWF monoclonal antibody, 95% of FVIII present in normal plasma remained bound to the Sepharose but only 78% of LE FVIII. In addition, the dissociation rate constant of the FVIII/vWF complex was higher for LE than for normal FVIII: the dissociation half-time of normal and LE FVIII/vWF complexes were 14 min and 4 min, respectively. In patient LE's plasma, the FVIII level measured by ELISA, indicating that LE FVIII molecules had a normal specific activity in coagulation assays and that the low FVIII level in the patient's plasma was due to either reduced synthesis or altered clearance of FVIII. Similar results were obtained with plasma of patient LE's hemophilic brother and one unrelated hemophiliac carrying the same mutation, Arg2150 -> His. Altogether, these results indicate that the C1 domain is involved in FVIII binding to vWF and suggest that the reduced affinity of FVIII for vWF contributes to the low levels of circulating FVIII in patients with mutation Arg2150 -> His.

Abstract# 2918

CHARACTERIZATION OF TWO NATURALLY OCCURRING MUTATIONS IN THE SECOND EGF-LIKE DOMAIN OF FACTOR VII. M. Hurtaud[a], A.A. Arbini[a], J.A. Carew[a], F. Peyvandi[b], K.A. Bauer. *Brockton West Roxbury VA Medical Center; Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA.*

We investigated the molecular basis underlying severe factor VII (FVII) deficiency in two Italian patients with moderate to severe bleeding disorders. Sequencing demonstrated that both were homozygous and had missense mutations in exon 5, which codes for the second EGF-like domain of FVII. Their FVII levels and genotypes (including the Arg353Gln and deoxynucleotide insert at nucleotide -323 in the 5'-flanking region) are tabulated below:

Missense Mutation	VII:Ag	VII:C	Arg353Gln	-323 Insert
G→T 7824, Gly97Cys	2%	<1%	+/+	+/+
G→T 7834, Gln100Arg	12%	<1%	-/-	-/-

Transient transfections were performed in COS-1 cells with wild type (wt) and mutant FVII cDNAs in the pED vector with measurement of FVII levels in cell lysates (L) and conditioned media (CM).

	FVIIwt (N=8)	FVIICys97 (N=8)	FVIIArg100 (N=8)
L VII:Ag	100 ± 2.8	38.1 ± 3.9	54.6 ± 3
CM VII:Ag	100 ± 3	6.6 ± 0.5	16.7 ± 2.1
CM VII:C	100 ± 2.9	6.1 ± 0.3	2.8 ± 0.2

The FVIICys97 and FVIIArg100 mutations result in defective secretion; the Arg100 mutation also affects FVII function since VII:C is lower than VII:Ag both in vivo and in vitro. Using stably transfected CHO cells, metabolic labeling studies demonstrated that FVIIwt in cell lysates was maximal 30-60 min after the pulse and decreased as FVII was secreted. FVIICys97 and FVIIArg100 persisted intracellularly for a longer period of time than FVIIwt (40% and 55% of the maximal amount of intracellular FVIICys97 and FVIIArg100, respectively, were still present 240 min after the end of the labeling period as compared to 12% for FVIIwt). Immunohistochemical studies showed that FVIIArg100 was present diffusely in the cytoplasm suggesting predominant localization in the endoplasmic reticulum, while FVIIwt and FVIICys97 stained mostly in the perinuclear area indicating its transit to the Golgi complex. Experiments using inhibitors of protein degradation suggested that FVIICys97 was degraded in a pre-Golgi, non-lysosomal compartment by a cysteine protease, while FVIIArg100 was retained in the endoplasmic reticulum for a longer period of time than FVIIwt. In CM under non-denaturing conditions, FVIIArg100 had a lower apparent molecular weight than FVIIwt which likely results from misfolding of the protein due to abnormal disulfide bond formation.

Abstract# 2919

THE ARG 2150 HIS MUTATION WITHIN THE FACTOR VIII C1 DOMAIN ELIMINATES A B CELL EPITOPE THAT IS PRESENT ONLY ON FACTOR VIII-VON WILLEBRAND FACTOR COMPLEXES. J.G.G. Gilles, R. Lavend'homme[a], K. Peerlinck[b], M. Jacquemin[a], M. Hoylaerts[a], S. Iorieux[a], C. Mazurier[c1], J. Vermylen[a1] and J.-M. Saint-Remy. *Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium; Laboratoire Français de Fractionnement et des Biotechnologies, Lille, France.*

A mild hemophilia A patient (LE) with an Arg2150His mutation in the C1 domain of the factor VIII (FVIII) light chain was shown to have anti-FVIII antibodies inhibiting allogeneic but not self FVIII in a one-stage coagulation assay. Additionally, the inhibitory activity of such antibodies was much increased in the presence of von Willebrand factor (vWF). Polyclonal anti-FVIII antibodies of this patient were purified by affinity adsorption using recombinant FVIII (rFVIII) or plasma-derived (pd) FVIII-vWF complexes. The rFVIII immunosorbent efficiently contained an inhibitory activity to allogeneic FVIII, but not to self FVIII with a titer comparable to that found in the total IgG fraction used for the adsorption procedure. The same IgG fraction sequentially passed through a pdFVIII and rFVIII-immunosorbent showed that adsorption on pdFVIII captured antibodies exhibiting split reactivity between wild-type FVIII and mutant Arg2150His FVIII, and that such antibodies were not retained by rFVIII immunosorbent. In addition, in ELISA, this fraction showed an important increased binding capacity to FVIII-vWF when compared to FVIII. These selectively inhibiting antibodies were shown to belong to the IgG2 isotype. To explain why vWF-dependent antibodies recognized wild-type but not self FVIII, the interaction between mutated FVIII and vWF was evaluated. The data indicated that a clinically-relevant FVIII B cell epitope generated by the association of Arg2150His mutation and receiving FVIII molecule. Patients carrying such an Arg2150His mutation are at risk of developing inhibitors to infusion of wild-type FVIII may therefore be at risk of developing inhibitors to allogeneic FVIII only. This point mutation in the FVIII light chain offers the first example of clinically-relevant FVIII B cell epitope(s) generated by the association of FVIII with vWF.

Abstract# 2920

INTRA- AND EXTRA-CELLULAR INSTABILITY OF A NOVEL Y283C MUTANT OF THE A SUBUNIT FOR COAGULATION FACTOR XIII CAUSED BY IMPAIRED PROTEIN FOLDING, DIMER FORMATION, AND HETEROTETRAMER ASSEMBLY. M. Souri[a], K. Kazui[a], T. Kaneshiro[a], K. Natsuaki[a], G. Catanzaro[a1], Y.C. Yee[a2] and A. Ichinose. *Department of Molecular Pathological Biochemistry and Biology, Yamagata University School of Medicine, Yamagata, Japan; [1]Department of Haematology, San Borroto Hospital, Vicenza, Italy; [2]Lerner Research Institute, Cleveland Clinic Foundation Cleveland OH 44195, USA.*

Coagulation factor XIII (XIII), a plasma transglutaminase, is a heterotetramer consisting of two catalytic A subunits (XIIIA) and two nonenzymatic B subunits (XIIIB). In the present study, a novel mutation in the XIIIA gene, Y283C, was identified in a patient with XIIIA deficiency. A recombinant Y283C protein was found to be labile when it was expressed in MEG-01 cells which can endogenously synthesize XIIIA. We also characterized in detail the Y283C mutant as well as two other mutants, G562R and I464stop, previously identified by Takahashi et al. (Blood 91: 2830-2838, 1998). All these mutants exhibited decreased thermostability and resistance against proteolytic cleavage when compared with a wild-type XIIIA. Gel-filtration analysis revealed that the mutants were in a monomer form, while the wild-type was in a dimer form. These results were consistent with the prediction by molecular modeling that the mutant molecules are misfolded and/or destabilized. Although assembly of a heterotetramer with XIIIB was demonstrated for Y283C by both gel-filtration analysis and a co-immunoprecipitation method using anti-XIIIB antibody, its binding ability was 10% that of the wild-type. No complex formation was observed for the G562R and I464stop mutants. The wild-type XIIIA was stabilized in plasma by the complex formation with XIIIB, and the XIIIA complexed with XIIIB was more resistant against proteolytic cleavage. On the other hand, mutant XIIIAs were unstable in plasma even in the presence of XIIIB. Thus, impaired folding, dimer formation, and heterotetramer assembly of the mutant XIIIAs lead to both intra- and extracellular instability, which is responsible for XIIIA deficiency in the patients.

Abstract# 2921

VON WILLEBRAND DISEASE TYPE 2M VICENZA IN ITALIAN AND GERMAN PATIENTS – THE FIRST CANDIDATE MUTATIONS. R. Schneppenheim[a], A.B. Federici, U. Budde[a], G. Castaman[a], R. Drewke[a], S. Krey[a], P.M. Mannucci, G. Rieger[a], F. Rodeghiero[a], B. Ziegler[a], R. Zimmermann[a]. *University Children's Hospital, Hamburg; University Children's Hospital Freiburg; Kurpfalzklinik, Heidelberg, Germany; Hemophilia and Thrombosis Center, University of Milan; Division of Hematology, Vicenza, Italy.*

Von Willebrand disease (VWD) type 2, is caused by structural and functional defects of the von Willebrand factor (vWF). VWD type 2M Vicenza is characterized by autosomal dominant inheritance, low vWF:Ag and the presence of "supranormal" multimers in plasma. In the formerly described Italian patients (Mannucci et al, Blood 71:65-70, 1988), the molecular defect is linked to the vWF gene (Randi et al., Thromb Haemost 69:173-176 1993). However, no specific mutations have been identified until now. As part of a mutation screening program

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